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αMSH and Cyclic AMP Elevating Agents Control Melanosome pH through a Protein Kinase A-independent Mechanism

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Melanins are synthesized in melanocytes within specialized organelles called melanosomes. Numerous studies have shown that the pH of melanosome plays a key role in the regulation of melanin synthesis. However, until now, acute regulation of melanosome pH by a physiological stimulus has never been demonstrated. In the present study, we show that the activation of the cAMP pathway by αMSH or forskolin leads to an alkalinization of melanosomes and a concomitant regulation of vacuolar ATPases and ion transporters of the solute carrier family. The solute carrier family members include SLC45A2, which is mutated in oculocutaneous albinism type IV, SLC24A4 and SLC24A5, proteins implicated in the control of eye, hair, and skin pigmentation, and the P protein, encoded by the oculocutaneous albinism type II locus. Interestingly, H89, a pharmacological inhibitor of protein kinase A (PKA), prevents the cAMP-induced pigmentation and induces acidification of melanosomes. The drastic depigmenting effect of H89 is not due to an inhibition of tyrosinase expression. Indeed, H89 blocks the induction of melanogenesis induced by LY294002, a potent inhibitor of the PI 3-kinase pathway, without any effect on tyrosinase expression. Furthermore, PKA is not involved in the inhibition of pigmentation promoted by H89 because LY294002 induces pigmentation independently of PKA. Also, other PKA inhibitors do not affect pigmentation. Taken together, our results strengthen the support for a key role of melanosome pH in the regulation of melanin synthesis and, for the first time, demonstrate that melanosome pH is regulated by cAMP and αMSH. Notably, these are both mediators of the response to solar UV radiation, the main physiological stimulus of skin pigmentation.

Melanins, the pigments responsible for skin and hair pigmentation in mammals, are synthesized through an enzymatic process, catalyzed by tyrosinase, Tyrp1 (tyrosinase-related protein 1), and dopachrome tautomerase, which converts tyrosine to melanin pigments (1). This process takes place in lysosome-related vesicles called melanosomes within melanocytes. These vesicles are transported to the dendrite tips and are transferred to the surrounding keratinocytes to ensure hair and skin pigmentation (2). In humans, melanins play a key photo-protective role against the noxious effect of solar UV radiation (3).

Numerous clinical observations, animal models, and experimental data clearly demonstrate that αMSH and the cAMP pathway are key physiologic regulators of skin and hair pigmentation in mammals, including humans (4). Keratinocyte-derived αMSH, which is increased by UV radiation, binds to the MCR1 (melanocyte melanocortin receptor type 1) (5) and increases the cAMP content in melanocytes. Cyclic AMP, through the activation of protein kinase A (PKA)[5] and CREB, up-regulates the expression of MITF, a key transcription factor that controls the expression of melanogenic enzyme, tyrosinase, Tyrp1, and dopachrome tautomerase (6). In melanocytes, cAMP activates the ERK pathway, which also plays a key role in melanin synthesis (7), at least in part through the regulation of MITF expression and stability (8, 9).

In addition to the control of melanin synthesis, cAMP controls other fundamental parameters of pigment production and processing by melanocytes. Indeed, cAMP favors melanosome maturation by controlling the expression of Silver (10) and OA1 (11). Cyclic AMP also induces dendrite formation (12) and stimulates the transport of melanosomes to the tips of dendrites (13) by controlling Rab27a expression (14).

Another key factor in melanogenesis is the pH of melanosomes. Numerous studies have shown that melanosomes, which are lysosome-related organelles, have an acidic pH (15). However, although some studies claim that this acidic pH is required for melanin synthesis (16), several other studies have demonstrated that vacuolar ATPase inhibitors, which increase melanosome pH, are able to stimulate melanin synthesis (17). Thus, taking into account the importance of pH, we sought to investigate the effect of αMSH and cAMP on the regulation of melanosome pH.

In the present study, we show that cAMP increases the pH of melanosomes and regulates the expression of several vacuolar ATPases and ion transporters, which might be important for

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[5] The abbreviations used are: PKA, protein kinase A; CREB, cAMP-response element-binding protein; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; V, vacuolar; MITF, microphthalmia associated transcription factor; DOPA, 3,4-dihydroxy-*L*-phenylalanine; DAMP, *N*-(3-((2,4-dinitrophenyl)amino)propyl)-*N*-(3-aminopropyl)-methylamine dihydrochloride.

the control of melanosome pH. *N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide (H89), prevents the alkalinization of melanosomes induced by cAMP-elevating agents and completely blocks melanin synthesis independent of PKA inhibition and regulation of tyrosinase expression. Taken together, our results further support the role of melanosome pH in the regulation of melanin synthesis. Additionally, these findings show, for the first time, that melanosome pH is regulated by αMSH and cAMP, which are induced as the consequence of physiological stimuli evoked by solar UV radiation.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids—The monoclonal antibody to Tyrp1 (B8G3) was a gift from Dr. Parson (Queensland Institute of Medical Research, Brisbane, Australia), anti-Silver (HMB45) monoclonal antibody was purchased from Dakopatts and anti-MITF (C5) was obtained from Abcam. The anti-ERK2 monoclonal antibody was purchased from Santa Cruz Biotechnology. DAMP- and fluorescein isothiocyanate-labeled anti-2,4-dinitrophenol were purchased from Invitrogen. The polyclonal anti-phospho-CREB was purchased from Cell Signaling Technology.

Cell Culture—The mouse melanoma cell line B16F10 was grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum, 100 units/ml, 50 μg/ml of penicillin-streptomycin and maintained in 5% of CO_2 at 37 °C.

Determination of Melanin Content—B16 cells (150×10^3 cells/35-mm well) were cultured for 36 h in the presence of different effectors. Cells were centrifuged, and pellets were photographed and then solubilized in 100 μl of 1 M NaOH in 70 °C for over 2 h to dissolve melanins. The relative quantity of melanin was estimated by the absorbance at 405 nm and correlated to the quantity of proteins. The results are expressed as -fold of stimulation compared with the control conditions.

Western Blot—B16 melanoma cells (200×10^3 cells/35-mm well) were stimulated by different effectors during the indicated time. Cells were then solubilized for 10 min at 4 °C in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 μM leupeptin, 1 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), 100 units/ml aprotinin, 10 mM NaF, and 1 mM Na_3VO_4. Then, 30 μg of protein was separated by electrophoresis on 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore). The membrane was saturated for 1 h at 25 °C in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20, 3% bovine serum albumin, and 5% gelatin. The primary antibody was incubated for 18 h at 4 °C. After three washes of 10 min in 10 mM Tris, pH 7.4, 150 mM NaCl, and 1% Triton X-100, the secondary antibody conjugated with horseradish peroxidase (Dakopatts) was incubated for 1 h at 25 °C. After three additional washes, proteins of interest were revealed by ECL (Amersham Biosciences).

Transfection and Dosage of Luciferase Activity—B16 melanoma cells (20×10^3 cells by 16-mm well) were transfected with Lipofectamine (Invitrogen). For each time point, 0.3 μg of the reporter plasmid pTyro or pMITF, as well as 0.05 μg of pCMVβgal, were incubated with 2 μl of Lipofectamine in 25 μl of OptiMEM (Invitrogen) for 20 min at 25 °C. The mixture was added to the B16 cells over a 6-h period and then replaced by Dulbecco's modified Eagle's medium, 7% fetal calf serum, in the presence of 20 μM forskolin and/or 5 μM H89. 36 h later, cells were solubilized and assayed for luciferase and β-galactosidase activities. All transfections were repeated several times using different plasmid preparations.

Measure of DOPA Oxidase Activity—B16 cells (200×10^3 cells/35-mm well) were cultured in the absence or presence of 20 μM forskolin and/or 5 μM H89. Proteins were solubilized for 3 min at 4 °C in 0.1 M phosphate buffer, pH 7.4, and 1% Triton X-100, and then 60 μg of protein was incubated with 200 μg of L-DOPA at 37 °C. The kinetics of the reaction were followed by measuring absorbance of the product formed (dopaquinone) at 570 nm.

Immunofluorescence Studies—B16 cells were cultured on glass slides in 24-mm wells at 15×10^3 cells/slide and treated, or not treated, for 36 h by 1 μM αMSH or 20 μM forskolin and/or 5 μM H89. Cells were washed with serum-free culture medium and incubated for 20 min in the presence of 30 μM DAMP. Cells were fixed in 3% paraformaldehyde for 20 min at 25 °C. Slides were washed with phosphate-buffered saline (PBS), incubated 10 min in NH_4Cl, and permeabilized in PBS with 0.1% Triton for 2 min. Slides were then incubated with a fluorescein isothiocyanate-labeled rabbit anti-2,4-dinitrophenol antibody (1/50 in PBS plus 1% bovine serum albumin). Melanosome labeling with primary monoclonal antibody (B8G3 anti-TYRP-1, 1/10; HMB45 anti-Pmel-17, 1/20) was performed in PBS plus 1% bovine serum albumin. After three washes with PBS, slides were incubated with Texas Red-labeled anti-mouse antibody (1/500). Cell labeling was observed using a confocal microscope (LSM510, Zeiss).

Electron Microscopy—B16 cells cultured with or without 20 μM forskolin and/or 5 μM H89 for 36 h were fixed in 2% glutaraldehyde for 3 h at 4 °C, followed by a post-fixation in 2% osmic acid for 1 h at 4 °C. Cells were subjected to successive dehydrations in alcohol baths, 30, 50, 75, and 95 °C for 15 min each; 100 °C, overnight. Following dehydration, samples were exposed to increasing amounts of Epon, specifically, Epon/alcohol (50/50) over 3 h, and then in Epon/alcohol (75/25), and finally in 100% Epon for 18 h. Cells were then embedded in 10% gelatin for 2 days in 60 °C. Sections of 700 Å were labeled with uranyl acetate and lead citrate.

Microarray Analysis—The oligonucleotide microarrays contained ~25,000 distinct oligonucleotide probes, covering most of the known human transcripts. The list of the probes is available on-line. RNAs were labeled using an amplification protocol, as described previously (18, 19). Briefly, 1 μg of total RNA was amplified with the Amino Allyl MessageAmp amplified RNA kit (Ambion, Austin, TX) according to the manufacturer's instructions. Cy3- and Cy5-labeled amplified RNA were fragmented with Ambion amplified RNA fragmentation reagents, purified, and then made up in Agilent hybridization buffer. Labeled cRNAs were then hybridized to human pan-genomic microarrays at 62 °C for 16 h. Microarrays were washed and scanned with a GenePix scanner (Axon Instruments, Molecular Devices Corporation, Union City, CA). TIF images containing the data from each fluorescence channel were quantified with the GenePix Pro 6.0 program (Axon Instruments). Normaliza-

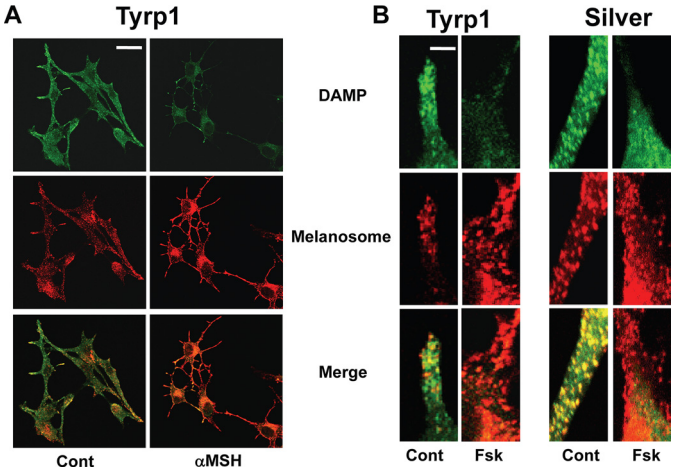

FIGURE 1. **Alkalinization of melanosomes by αMSH and forskolin.** B16 mouse melanoma cells were incubated with or without αMSH (αMSH or control (*Cont*), respectively) (*A*) or 20 μM forskolin (*Fsk*) (*B*). After 36 h, cells were incubated with 30 μM DAMP for 30 min. After fixation in 3% paraformaldehyde and permeabilization with 0.1% Triton X-100, DAMP was visualized with fluorescein isothiocyanate-labeled anti-2,4-dinitrophenol, and melanosomes were labeled with either anti-Tyrp1 (B8G3, *A* and *B*) or anti-Silver (HMB45, *B*) and a secondary Texas Red anti-mouse antibody. *A*, *scale bar* = 20 μm. *B*, higher magnification images. *Scale bar* = 3 μm.

tion and statistical analysis were performed using the limma package available from Bioconductor.

RESULTS

αMSH Increases pH of Melanosomes—First, using DAMP, a weak base that accumulates in acidic compartments, we evaluated the effect of αMSH on the pH of melanosomes. Melanosomes were labeled with an antibody against Tyrp1, which is found in mature melanosomes. Under control conditions, there was significant DAMP labeling (*green*). Most of the Tyrp1-positive melanosomes, labeled in *red*, were also positive for DAMP and appeared *yellow* on the merged images. These observations indicate that, under control conditions, melanosomes are acidic. In cells treated with αMSH, DAMP expression decreased, and in merged images, melanosomes appeared *red* (or *orange*), indicating a change of melanosome pH (Fig. 1*A*). At higher magnification, identical results were seen following forskolin treatment, which increases cAMP content (Fig. 1*B*). Using antibodies to Silver, to visualize early melanosomes, or to Tyrp1, to label mature melanosomes, we observed that both early and mature melanosomes appeared *yellow* under control conditions. Treatment with forskolin decreased DAMP labeling, and melanosomes appeared *red* (or *orange*), confirming that αMSH and cAMP induces an alkalinization of the melanosome milieu.

cAMP Regulates Expression of Vacuolar ATPases and Ionic Transporters—It is well known that melanosomes contain vacuolar ATPases and that inhibition of these ATPases leads to alkalinization of the melanosome, favoring melanin synthesis. Thus, we asked whether cAMP might modulate the expression of ATPases, in particular, vacuolar ATPases. Analysis of a DNA microarray showed that, among the 87 ATPases present in the array, 76 were significantly expressed in B16 melanoma cells. Eleven ATPases, including seven vacuolar ATPases (ATP6V0-B, -C and -D1; ATP6V1-A, -B2, -C1, and

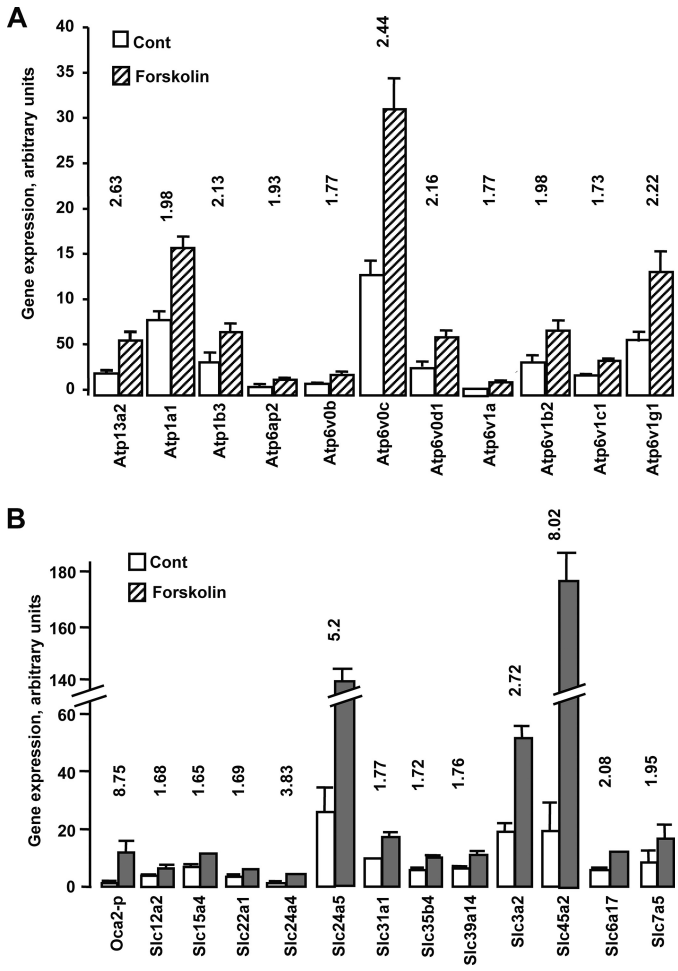

FIGURE 2. **cAMP regulates the expression of vacuolar ATPases and ionic transporters.** B16 mouse melanoma cells were incubated with or without (control) 20 μM forskolin for 36 h. RNA was purified, labeled, and analyzed on a pan-genomic mouse DNA microarray. *A*, ATPase expression was up-regulated after forskolin treatment by more than 70% ($p < 0.05$). *B*, SLC and P protein expression was stimulated after forskolin treatment by more than 70% ($p < 0.05$). -Fold change of stimulation is displayed above each *histogram*. *Cont*, control.

-G1), were significantly up-regulated by cAMP. Another ATPase, ATP6AP2, which is associated with lysosomes and possibly with melanosomes, was also up-regulated by cAMP (Fig. 2*A*). None of these ATPases were inhibited by cAMP.

As it has been reported that ATPase inhibitors induced alkalinization of the melanosome (20), the augmentation of ATPases should result in a decrease in melanosome pH. Thus, we examined the effect of cAMP on the expression of the membrane transporters of the SLC family that participate in the regulation of the electric and ionic equilibrium. Among the 335 SLC genes on our array, 199 were significantly expressed. Thirteen were up-regulated by cAMP, including SLC24A4, SLC24A5, and SLC45A2, all of which are involved in pigmentation. SLC6A6 and SLC9A3R1 were down-regulated by more than 50% (data not shown). Additionally, cAMP regulated the expression of OCA2, a transporter that plays a key role in pigmentation, but its function is not clearly identified (Fig. 2*B*). The localization of these ATPases and solute carriers to the melanosomes as well as the mechanisms by which this modulates melanosome pH remain to be elucidated. Neverthe-

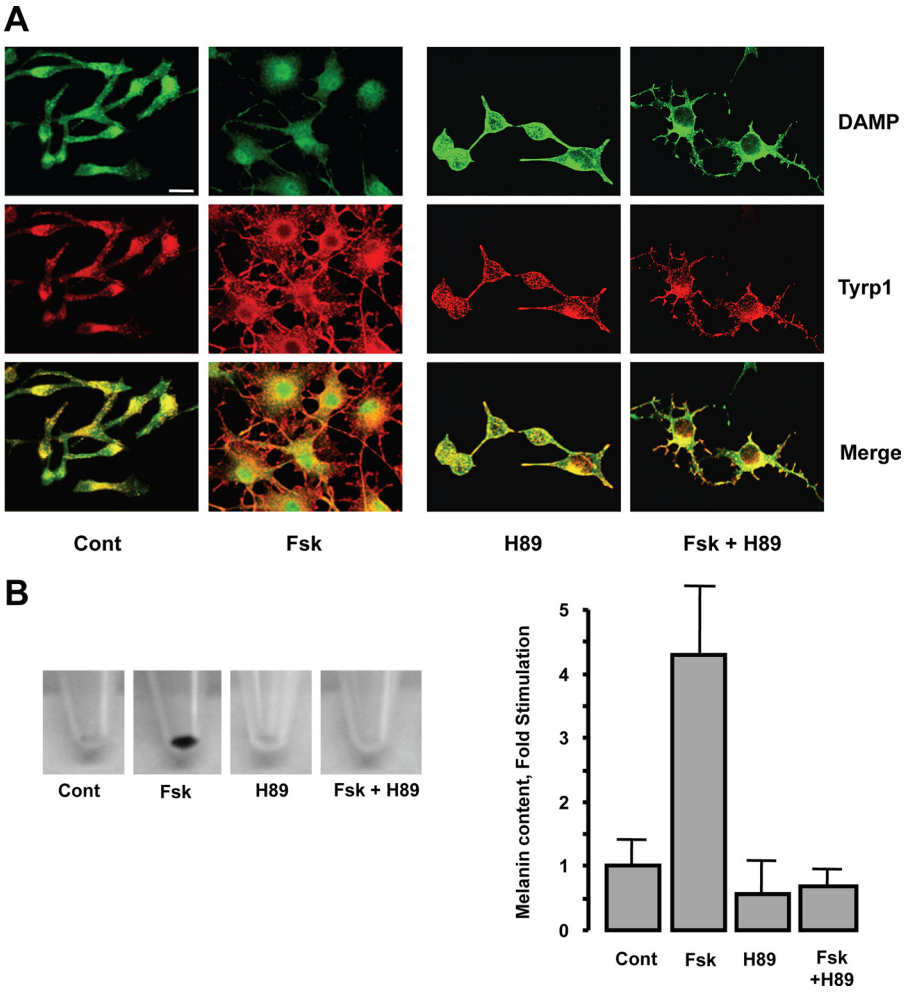

FIGURE 3. **H89 prevents cAMP-induced melanosome alkalinization and melanin synthesis.** B16 mouse melanoma cells were incubated with or without 20 μM forskolin (*Fsk* or control, respectively), in the presence or absence of 5 μM H89 for 36 h. *A*, cells were incubated with 30 μM DAMP for 30 min. After fixation in 3% paraformaldehyde and permeabilization with 0.1% Triton X-100, DAMP was visualized with fluorescein isothiocyanate-labeled anti-DNP, and melanosomes were labeled with anti-Tyrp1 (B8G3). *Scale bar* = 20 μm. *B*, representative cell pellets treated with vehicle (*Cont*), with forskolin, with H89 or with forskolin plus H89 (*Fsk* + *H89*) are displayed on the *left panel*. The melanin content, measured by spectrometry at 405 nm and normalized by the protein content, is shown on the *right panel*.

less, the data presented above indicates that cAMP may greatly influence ionic equilibrium in melanocytes and in melanosomes.

H89, a Pharmacological PKA Inhibitor, Prevents cAMP-induced Melanosome Alkalinization and Melanin Synthesis—Next, we investigated how cAMP regulates melanosome pH and the possible involvement of PKA. To do so, we examined the effect of H89, a PKA inhibitor, on the regulation of melanosome pH. We found that H89 prevented the cAMP-induced decrease in DAMP labeling compared with control conditions. When the cells were exposed to H89, the melanosomes, identified by Tyrp1 labeling, appeared *yellow* and thus acidic (Fig. 3*A*). Interestingly, H89 completely blocked melanin synthesis, as shown by the picture of the cell pellets and by the quantification of melanin content (Fig. 3*B*). Because PKA, through the control of MITF expression, regulates the transcription of the tyrosinase gene, the inhibition of melanin synthesis might be the result of the inhibition of tyrosinase expression. Therefore, we studied the effect of H89 on tyrosinase, Tyrp1, Silver, and MITF expression. H89 affected neither basal nor cAMP-induced expression of Tyrp1 or Silver. On the other hand, H89 clearly reduced the cAMP-induced up-regulation of tyrosinase and MITF expression (60% decrease). Additionally, H89 completely blocked the effect of cAMP on CREB phosphorylation (Ser-133), demonstrating that H89 had, indeed, inhibited PKA (Fig. 4*A*). Then, regulation of *Tyr* or *Mitf* transcription by H89 was evaluated by a luciferase assay. Although H89 reduced the effect of cAMP on the tyrosinase and MITF promoter, it did not completely prevent the activation of these promoters by cAMP (Fig. 4*B*). Finally, treatment of the cells with H89 led to a 40% decrease in the DOPA oxidase activity of tyrosinase (Fig. 4*C*). The addition of H89 *in vitro*, directly into the enzymatic assay, did not affect tyrosinase activity. This indicated that H89 is not a direct inhibitor of the DOPA oxidase activity of tyrosinase. Taken together, these observations indicate that H89 partially prevents the up-regulation of tyrosinase transcription and expression elicited by cAMP. However, it remained to understand why H89 led to the complete inhibition of melanin synthesis, while the level of tyrosinase in cells treated by H89 stayed clearly above the level of tyrosinase in basal cells.

H89 Affects Melanosome Structure—Next, we evaluated the effect of H89 on melanosome structure by electron microscopy. Under control conditions, we mainly observed the presence of round and slightly striated melanosomes, corresponding to type II melanosomes. After treatment with forskolin, elliptical black vesicles appeared, in which longitudinal striations were barely visible due to melanin accumulation. These vesicles resembled type III and IV melanosomes. In both control and forskolin-treated cells, H89 treatment resulted in the appearance of large round and vacuolized melanosomes resembling late endosomes (Fig. 5).

H89 Can Block Melanogenesis without Affecting Tyrosinase Expression—One might find intriguing that the inhibition of PKA does not lead to a more dramatic effect on tyrosinase expression. However, we have shown previously that, in addition to PKA, cAMP regulated other signaling pathways in melanocytes. Specifically, cAMP inhibited the PI 3-kinase pathway; the inhibition of this pathway by LY294002 resulted in a significant increase in tyrosinase expression and a stimulation of melanogenesis (21). Thus, we evaluated the consequence of

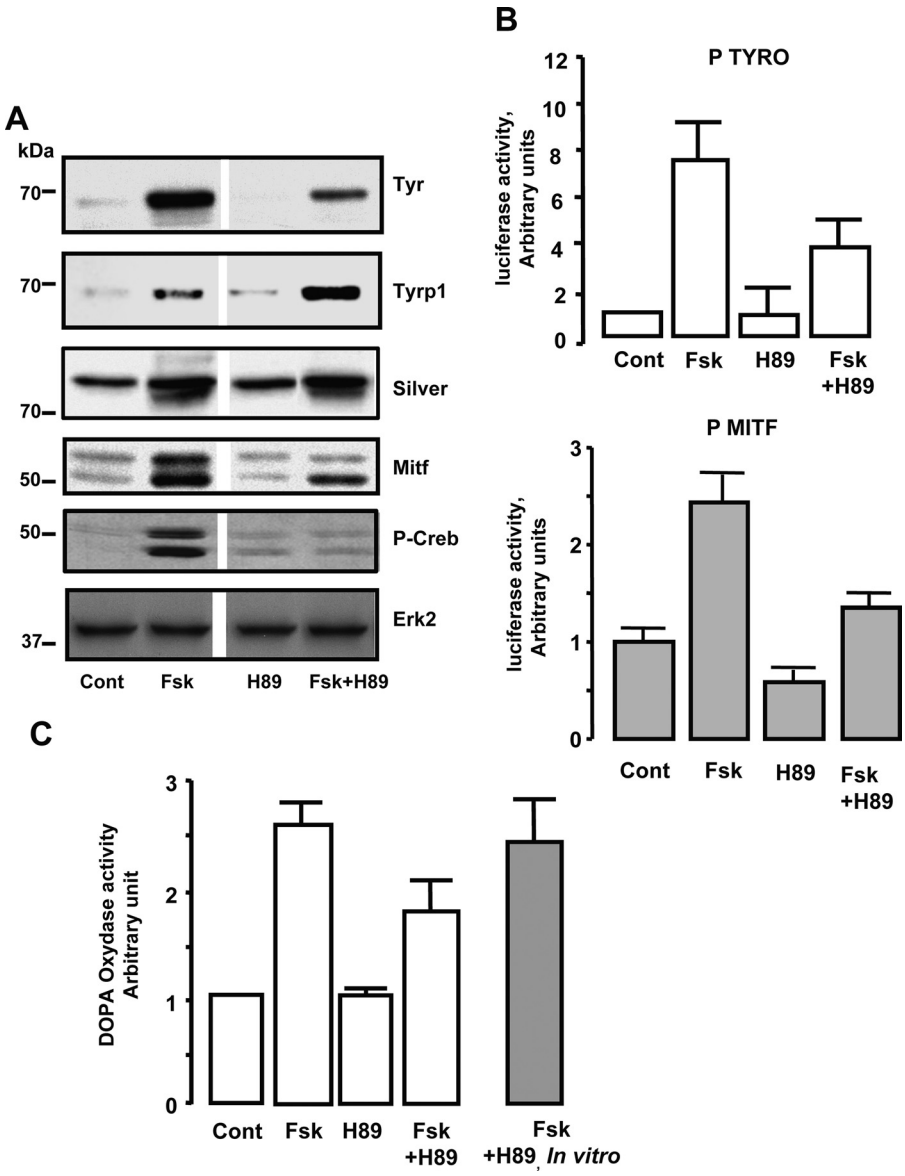

FIGURE 4. **Effect of H89 on tyrosinase expression and activity.** *A*, B16 mouse melanoma cells were incubated with or without 20 μM forskolin (*Fsk* or control, respectively), in presence or absence of 5 μM H89 for 36 h. Cells were solubilized, and proteins were analyzed by Western blot using antibodies against tyrosinase (*Tyr*), Tyrp1, Silver, Mitf, and Phospho-CREB (*P-Creb*) and, as loading control, Erk2. *B*, B16 mouse melanoma cells were transfected with a reporter plasmid, encoding luciferase under the control of either the tyrosinase promoter (*P TYRO*) or MITF promoter (*P MITF*). Cells were either mock-treated (control) or treated with 20 μM forskolin, in the presence or absence of 5 μM H89 for 36 h, and then solubilized and assayed for luciferase activity. *C*, B16 mouse melanoma cells were incubated with or without 20 μM forskolin (*Fsk* or control) in the presence or absence of 5 μM H89 for 36 h. Solubilized proteins were incubated with L-DOPA and assayed for DOPA oxidase activity by measuring the OD at 570 nm. To evaluate the direct effect of H89 on the DOPA oxidase activity of tyrosinase, H89 was added with L-DOPA from forskolin-treated cells (*In vitro*). *Cont*, control.

H89 treatment on the effects of LY294002 on tyrosinase and MITF expression as well as on melanin synthesis. As shown in Fig. 4, H89 clearly inhibited the expression of tyrosinase and, to a lesser extent, MITF in cAMP-treated cells. Inhibition of the PI 3-kinase by LY294002 increased the expression of tyrosinase and MITF as efficiently as cAMP. In contrast, treatment with H89 did not affect MITF expression and only slightly reduced the tyrosinase level (Fig. 6*A*). Remarkably, the strong stimulation of melanin synthesis induced by LY294002 was completely inhibited by H89 (Fig. 6*B*). This observation emphasizes the lack of correlation between the tyrosinase level and melanin synthesis in H89-treated cells, suggesting that the H89-induced loss of melanogenesis is not the direct consequence of a reduction of tyrosinase expression or of PKA inhibition.

Neither PKA nor Other Kinases Inhibited by H89 Are Involved in Inhibition of Melanogenesis—To further investigate the role of PKA inhibition in the effect of H89, we examined several other pharmacological inhibitors of PKA including H7, H8, H9, and HA1077. In preliminary studies, we have shown that the maximal inhibition of melanin synthesis with less than 5% decrease in cell viability was obtained using 5 μM of H89 (data not shown). Therefore, we compared the effect of 5 μM H89 to the other PKA inhibitors (Fig. 7*A*). H89 dramatically reduced melanin synthesis (95% inhibition). However, H7, H8, H9, and HA1077 failed to inhibit melanogenesis. Notably, H7, H8, and H9 were used at 50 μM; higher concentrations of H7 and H9 led to a 95% and 80% decrease in cell viability, respectively. H8 is not soluble at concentrations above 50 μM. HA1077 slightly inhibited (20%) melanin synthesis at 10 μM; at a higher concentration (50 μM), melanin synthesis decreased by 40%, but there was a 25% decrease in cell viability. H7, H8, H9, and HA1077 have been used at concentration markedly above the IC_{50} for PKA (0.5, 2, 1.3, and 1 μM, respectively) (22, 23), and they indeed inhibited CREB phosphorylation (data not shown). However, they did not affect melanin synthesis. This indicates that PKA inhibition is not sufficient for the inhibition of melanogenesis by H89.

H89 is the most specific inhibitor of PKA, but it is also able to inhibit other kinases (24) (Table 1) including S6 kinase, AKT, MSK1, ROCK-II, and MAPKAPK1. Therefore, we evaluated the effects of pharmacological inhibitors of these kinases on melanin synthesis (Fig. 7*B*). These inhibitors did not affect the melanogenic effect induced by forskolin. As reported previously, the inhibition of MEK, which is required for MAPKAPK1 activation, AKT, S6 kinase, and ROCK-II by PD98059 (25), LY294002, rapamycin (21), and Y27632 (12), respectively,

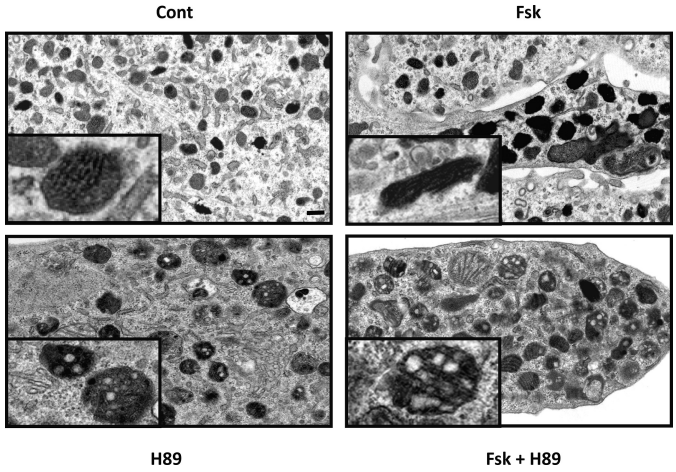

FIGURE 5. **H89 affects melanosome structure.** Shown are electron micrographs of B16 mouse melanoma cells incubated with or without 20 μM forskolin (*Fsk* or control, respectively) in the presence or absence of 5 μM H89 for 36 h. *Scale bar* = 1 μm. A higher magnification of melanosomes is shown in the *lower left corner*. *Cont*, control.

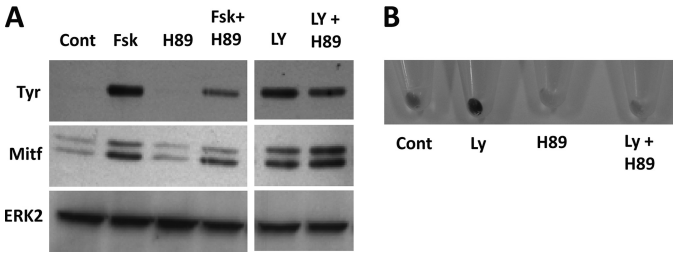

FIGURE 6. **H89 blocks the melanogenic effects of LY294002 without inhibition of tyrosinase expression.** B16 mouse melanoma cells were incubated with or without 20 μM forskolin or 5 μM LY294002 (*Fsk*, *LY*, or *Cont*, respectively), in the presence or absence of 5 μM H89 for 36 h. *A*, cells were solubilized, and proteins were analyzed by Western blotting using antibodies against tyrosinase (*Tyr*), Mitf and, as a loading control, ERK2. *B*, representative cell pellets are shown.

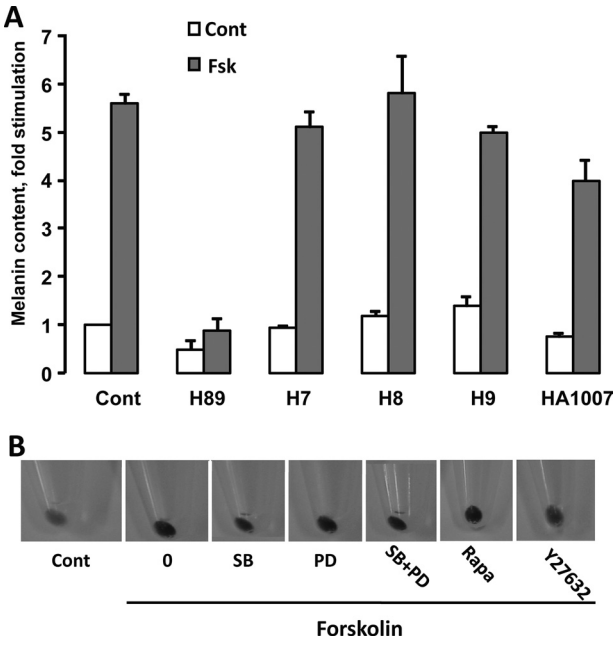

FIGURE 7. **Neither PKA nor other kinases inhibited by H89 mediate the depigmenting effects of H89.** *A*, B16 mouse melanoma cells were incubated with or without 20 μM forskolin (*Fsk*), in the presence of different PKA inhibitors (5 μM H89, 50 μM H7, 50 μM H8 and 50 μM H9, and 10 μM HA1007) for 36 h. Melanin content was measured by spectrometry at 405 nM and normalized by the protein concentration. *B*, B16 mouse melanoma cells were incubated with or without 20 μM forskolin, in the presence of different kinase inhibitors known to be inhibited by H89 for 36 h (SB202190 (*SB*), PD98059 (*PD*), Y27632, and rapamycin (*Rapa*)). Representative cell pellets are shown. *Cont*, control.

TABLE 1
IC_{50} of H89 for different kinases
Data are from Davies *et al.* (24).

Kinase	IC_{50}
S6K1	80 nM
MSK1	120 nM
PKA	135 nM
ROCK-II	270 nM
AKT	2.6 μM
MAPKAPK1	2.8 μM

resulted in a marked stimulation of melanin synthesis. Inhibition of MSK1 activation by the p38 inhibitor SB202190 did not affect melanogenesis (data not shown). Taken together, these observations indicate that none of these kinases is involved in the H89-mediated inhibition of melanogenesis.

DISCUSSION

In the present study, we demonstrated that the activation of the cAMP pathway by αMSH or forskolin led to alkalinization of melanosomes. Our results are in agreement with previous reports demonstrating that melanin synthesis was stimulated by agents that increase melanosome pH. This is true for the lysosomotropic compound, ammonium chloride, ionophores such as nigericin and monensin, and inhibitors of the vacuolar ATPases such as bafilomycin (26, 27). Fuller *et al.* (28) reported that the melanosomes of melanocytes from Caucasian skin displayed low tyrosinase activity and were more acidic than the melanosomes of melanocytes from black skin, which had high tyrosinase activity. Our observation provides a physiological link between melanosome pH and extracellular signals that trigger melanocyte differentiation. These findings indicate that melanosome pH is not only dictated by genetic determinants but that it can also be subjected to physiological environmental regulation, such as UV exposure that regulates αMSH production by keratinocytes. These observations can be extended to human melanocytes because we observed that (i) the amount of visible melanin was markedly increased when melanocytes from Caucasians were cultured in medium favoring melanosome alkalinization, and (ii) H89 decreased melanin content in melanocytes from black skin (data not shown).

The mechanisms and molecular players involved in the regulation of melanosome pH remain to be elucidated. Of course, the main player in this process is vacuolar (V)-ATPase itself; its presence in melanosomes has been demonstrated by proteomic analysis (29), and its role has been established by the use of a specific inhibitor. In line with these observations, our results demonstrate that the regulation of the expression of several V-ATPase subunits by cAMP strengthens the involvement of this proton pump in the physiologic regulation of melanosome pH. Notably, cAMP up-regulates the expression of the V-ATPase sub-units, which should result in the acidification of the melanosome. However, the disruption of the equilibrium between the different V-ATPase subunits might affect the assembly and/or dissociation of the V-ATPase V1 (ATP hydrol-

ysis) and V0 (proton translocation) complexes, which is the main mechanism of regulation of V-ATPase activity (30). V-ATPases are a key component in the regulation of the organelle and more specifically of melanosome acidification. However, the pH of an organelle is also greatly influenced by the internal ionic equilibrium. This ionic equilibrium is mainly controlled by ion pumps, such as Na^+/K^+-ATPase, and ion-specific channels, such as chloride, potassium, and sodium channels (30). The role of these ion pumps and channels in melanosomes has not yet been investigated.

Furthermore, ion exchange through the vast family of solute carriers (SLC) might also play a pivotal role in the regulation of melanosome pH. Among them, SLC45A2, formerly MATP or AIM1, is located in melanosomes and is mutated in oculocutaneous albinism type IV (31). Additionally, the potassium-dependent sodium/calcium exchangers SLC24A4 and SLC24A5 have been implicated in the control of eye, hair, and skin pigmentation (32–34). SLC24A5 has been detected in melanosomes (33) and might participate in the control of the ionic equilibrium and pH of these organelles. SLC45A2, SLC24A5, and SLC24A4 were strongly up-regulated in forskolin-treated cells, further supporting their role in the control of pigmentation. SLC3A2, a neutral amino acid transporter, whose function in pigmentation is not known, was also found in melanosomes by a proteomic approach (35), and its expression was markedly stimulated by forskolin.

Finally, P protein, encoded by the OCA2 gene, which is mutated in the oculocutaneous albinism type II, was stimulated by cAMP. This protein is related to the sodium/sulfate transporter of the SLC13 family. Initially, the P protein was thought to be involved in tyrosine transport. However, melanocytes isolated from pink-eyed dilution mice, which have a mutation in the P gene that results in the lack of the P protein, have normal tyrosine transport. The P protein was also implicated in the control of melanosome pH. Indeed, melanosomes in melanocytes from pink-eyed dilution mice were reported to have a less acidic pH that did not favor melanin synthesis (16, 36). However, these observations are not in agreement with either the numerous studies demonstrating that melanogenesis is stimulated by agents that increase melanosome pH (17, 26–28) or with our present results showing that cAMP increases melanogenesis and melanosome pH. The exact role of these pumps, channels, and exchangers in the regulation of melanosome pH and pigmentation need to be further investigated.

Most of the effects of cAMP are mediated by PKA. However, we have shown previously that cAMP can act independently of PKA (7). Thus, we examined whether the effect of cAMP is mediated by PKA. We found that H89, a potent PKA inhibitor, altered melanosome structure and blocked the alkalinization of the melanosome elicited by cAMP. Concomitantly, we observed a complete inhibition of melanin synthesis, supporting the role of pH in melanogenesis. PKA also controls the expression of tyrosinase through MITF; thus, the effect of H89 might be ascribed to an inhibition of tyrosinase expression rather than to an acidification of the melanosome. However, the effect of H89 on MITF and on the tyrosinase promoter activity or protein expression was not as drastic as its effect on melanin synthesis. Further, H89 also inhibited the melanogenic effect of LY294002, an inhibitor of the PI 3-kinase. In this case, H89 did not significantly affect the expression of MITF or tyrosinase. Taken together, these observations indicate that H89 can block melanogenesis independent of an inhibition of tyrosinase expression/activity and supports the role of melanosome acidification in the anti-melanogenic effect of H89. Interestingly, H89 altered the structure of melanosomes that resemble late endosomes, suggesting that H89 might block the melanosome maturation. This observation links melanosome pH to the melanosome biogenesis process.

Notably, the induction of melanogenesis by LY294002 does not involve the activation of PKA, suggesting that H89 might affect melanogenesis but not through inhibition of PKA. This hypothesis was confirmed by the use of several PKA inhibitors, H7, H8, H9, and HA1077 that were unable to affect melanin synthesis despite being used at concentrations higher than the IC_{50} for PKA. Examination of another set of kinase inhibitors, including PD98059, SB202190, Y27632 rapamycin, and LY294002, demonstrated that the others kinases inhibited by H89 (S6K1, MSK1, ROCK-II, AKT, and MAPKAPK1) (24) were not involved in the inhibition of melanogenesis by H89. The molecular target for H89 that mediates its effect on melanogenesis and on melanosome pH is still unknown. However, it has been reported recently that H89 can act independently of its action on kinases (37). Indeed, H89 was reported to be a β-adrenergic receptor antagonist and to directly inhibit a potassium (Kv 1.3) and sodium (Na^+) channel. Thus, H89 can affect melanosome pH through a direct modulation of ion channels, leading to the inhibition of melanogenesis.

To summarize, the regulation of numerous ATPases and transporters by cAMP indicates that physiological stimuli evoked by solar light, αMSH and cAMP, may lead to extensive changes in the ionic equilibrium of melanocytes, resulting in the alkalinization of melanosomes. Because it has been clearly demonstrated that low pH impairs tyrosinase enzymatic activity (17), alkalinization would stimulate tyrosinase activity and favor melanin synthesis. Furthermore, the pH of organelles is essential for correct protein and vesicular trafficking (38). Particularly, several pigmentation defects (OCA2 and OCA4) were shown to be the consequence of incorrect tyrosinase targeting (39, 40). Thus, any modifications in melanosome pH might lead to changes in tyrosinase trafficking and in the regulation of melanogenesis. Finally, the strong inhibition of melanin synthesis and the acidification of melanosomes evoked by H89 indicate that this compound, as well as any agents that decrease melanosome pH are potential ingredients in the treatment of hyperpigmentation diseases such as melasma and lentigo senilis.

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